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FINAL TECHNICAL PERFORMANCE REPORT

GRANT #: N00014-02-1-0643

PRINCIPAL INVESTIGATOR: Jay B. Dean, Ph.D. (e-mail: jay.dean@wright.edu)

INSTITUTION: Wright State University

GRANT TITLE: Hyperbaric Imaging Equipment: Fluorescence Microscopy for In Vitro Studies of Oxygen Toxicity

AWARD PERIOD: 17 May 2002 through ~~9~~ 30 September 2003 (includes no-cost extension)

OBJECTIVE: To develop new instrumentation for hyperbaric research at the cellular level; specifically, to establish two (2) normobaric/hyperbaric fluorescence microscopy stations for imaging single cells and measuring membrane potential (V_m), intracellular pH (pH_i) and intracellular and extracellular levels of reactive oxygen species (ROS) in *in vitro* preparations of the rodent CNS and pulmonary system exposed to hyperbaric oxygen (HBO).

APPROACH: This is a novel approach to studying cellular mechanisms of cellular oxygen toxicity. It expands the scope of our ongoing ONR project three ways: 1) by enabling us to visualize single neurons with infra-red video microscopy for electrophysiological recording; 2) by enabling us to visualize neurons loaded with fluorescent dyes for measuring pH_i, ROS and other important bioactive substances; and 3) by enabling us to visualize pulmonary cells in lung tissue slices for parallel studies of pulmonary oxygen toxicity in conjunction with CNS oxygen toxicity.

The fluorescence microscopy laboratory consists of two complementary research stations. **Microscope #1** is a circa 1966 Bethlehem hyperbaric chamber (purchased from Riemers, Hyperbaric Chamber Clearing House), which was reconditioned and upgraded for conducting ratiometric fluorescence microscopy and electrophysiology in single cells in tissue slices during HBO. To accomplish this, new electrophysiology equipment was combined with a pre-existing hyperbaric fluorescence imaging system. This arrangement allows us to individually or simultaneously measure V_m (micropipette) and/or pH_i or ROS (fluorescent probes) in the same cell exposed to HBO, antioxidants, *etc*, to directly correlate changes in cell function (V_m, membrane conductance, firing rate, synaptic transmission) with potential oxygen-dependent stimuli (ROS) or signaling molecules (CO_2/H^+, Ca^{2++}) during HBO. **Microscope #2** is an optical pressure chamber, equipped with confocal/fluorescence microscopy equipment, which will be used to measure pH_i and ROS in individual cells labeled with fluorescent probes and exposed to normobaric hyperoxia and HBO. Imaging data are collected under the same conditions used for electrophysiology studies in our ongoing ONR research project.

ACCOMPLISHMENTS (throughout award period): We have purchased, built and assembled all of the necessary equipment items that were proposed in the original DURIP application. Currently, both microscopy stations are on-line and being used almost daily for cellular research pertaining to our ONR-funded project. Initially, several technical difficulties were encountered to get both imaging systems to work; however, these have been resolved and we are gathering data on a regular basis. Some of these data have been used in studies that were published

during 2003-04 (see below) and other studies are ongoing. Currently, the new equipment is being used to train four (4) Wright State University graduate students (1 Ph.D., 3 M.S.) working on our ONR-funded project. This summer a new ONR-funded postdoctoral fellow (Dr. Dominic D'Agostino) will join our lab and use this equipment. In addition, future studies are planned with a visiting scientist from the NMRC (PI, Lt. Shawn Soutiere, Ph.D.) and the PI of an ONR-funded project at Yale University (Dr. Walter Boron).

Microscope #1—We have successfully built, tested and used the epifluorescence microscope at >1 to 5 ATA helium and HBO. In addition, we have determined that the infra-red video microscopy works at hyperbaric pressure. Using the 2x microscope objective, we can resolve pyramidal cell layers in a tissue slice of rat CA1 hippocampus for studies of synaptic transmission during HBO {Garcia III, 2003 #515}. Using the 40x microscope objective, we can resolve single neurons in brainstem and hippocampal slices for electrophysiological recording (Dean et al., in preparation). Our initial studies have focused on the effects of HBO on synaptic transmission in the hippocampus and pH_i. Our electrophysiological studies in the CA1 hippocampus show that HBO increases both excitatory synaptic transmission and postsynaptic membrane excitability. At the highest doses of HBO a seizure-like response was observed. Preliminary measurements of nitric oxide (one type of ROS) indicate that NO levels are proportional to the level of oxygen at normobaric and hyperbaric pressures. Please refer to progress report for ONR Award No. N00014-04-1-0172 (reporting period: June 1, 2003-May 31, 2004) for additional details of experiments conducted using this DURIP-supported equipment.

Microscope #2—We have built the optical pressure and have setup the confocal/ fluorescence microscope. Currently, the confocal/fluorescence microscope is being used for a study of the effects of normobaric oxygen on pH_i in the brainstem. The optical pressure chamber will be used when the new ONR-supported postdoctoral fellow arrives this summer (Dr. D'Agostino). Our experiments indicate that pH_i is proportional to normobaric oxygen; that is, as tissue O_2 pressure increases so does intracellular pH. At 0.95 ATA O_2, the pH_i plateaus and then further oxygenation under conditions of HBO causes a decrease in pH_i. The measurements under conditions of HBO are preliminary, however, the picture that is emerging is that pH_i vs. oxygen level yields a "$\cap$-shaped" relationship. Additional research will be done to confirm this interesting finding. Please refer to progress report for ONR Award No. N00014-04-1-0172 (reporting period: June 1, 2003-May 31, 2004) for additional details of experiments conducted using this DURIP-supported equipment.

CONCLUSIONS: We have determined that it is technically feasible to use a microscope under hyperbaric conditions to visualize cells for electrophysiological recording and/or fluorescence microscopy imaging. To our knowledge, this is the first time that these types of experiments have been conducted under hyperbaric conditions.

SIGNIFICANCE: The use of cutting-edge cellular research tools under hyperbaric conditions has always been a technical challenge. This project, however, demonstrates that with the appropriate equipment that cutting edge microscopy techniques can be adapted to hyperbaric research. This is expected to assist investigators, especially ONR-funded researchers, who are interested in the cellular mechanisms of oxygen toxicity of the CNS and pulmonary systems. Likewise, it is anticipated that these tools will be useful for studies of high-pressure nervous syndrome, nitrogen narcosis and the anesthetic effects of gases on the CNS.

PATENT INFORMATION: Presently, we are exploring the procedures for patent application with the Office of Research and Sponsored Programs at Wright State University.

AWARD INFORMATION: During the course of this research project, the PI (Dr. Jay B. Dean) was named the Brage Golding Distinguished Professor of Research at Wright State University (http://www.wright.edu/ cgibin/news_item.cgi?514). Dr. Dean also was promoted to Acting-Chair of the Department of Anatomy and Physiology and was named to a study section at the National Institutes of Health (Respiratory and Integrative Biology and Translational Research, RIBT).

In addition, Mr. Alfredo J. Garcia III (WSU Biomedical Sciences Ph.D. candidate), who uses Microscope #1 for his dissertation research, was invited to speak at a Featured Topic symposium at the 2004 Experimental Biology meeting in Washington DC. Further, Mr. Garcia received the top graduate student presentation award at the 2004 Miami Valley Chapter of the Society for Neuroscience Meeting in Cincinnati, Ohio, for research he conducted using Microscope #1.

REFEREED PUBLICATIONS (for total award period): The research equipment built with this DURIP award was used in research that produced six (6) papers published during 2003-04.

1. Dean, J.B., Mulkey, D.K., Garcia III, A.J., Putnam, R.W., and Henderson III, R.A. (2003) Neuronal sensitivity to hyperoxia, hypercapnia, and inert gases at hyperbaric pressures. J. Appl. Physiol. 95(3): 883-909.
2. Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Hyperbaric oxygen and chemical oxidants stimulate CO_2/H^+-sensitive neurons in rat brain stem slices. J. Appl. Physiol. 95 (3): 910-921.
3. Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Pressure ($\leq$4 ATA) increases membrane conductance and firing rate in the rat solitary complex. J. Appl. Physiol. 95(3): 922-930.
4. Dean, J.B., Mulkey, D.K., Henderson III, R.A., Potter, S.J., and Putnam, R.W. (2004) Hyperoxia, reactive O_2 species, and hyperventilation: oxygen-sensitivity of brain stem neurons. J. Appl. Physiol. 96: 784-791.
5. Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2004) Oxidative stress decreases intracellular pH, Na^+/H^+ exchange and increases excitability of solitary complex neurons from rat brain slices. Am. J. Physiol. Cell Physiol. 286: C940-C951.
6. Mulkey, D.K., Henderson III, R.A., Ritucci, N.A., Putnam, R.W., and Dean, J.B. (2004) Chemical oxidants acidify solitary complex (SC) neurons in rat. Undersea Hyperbaric Med. 31(1): 107-111.

BOOK CHAPTERS, SUBMISSIONS, ABSTRACTS AND OTHER PUBLICATIONS (for total award period): The research equipment built with this DURIP award was used in research that produced 10 abstracts for national meetings during 2002-04.

1. Garcia III, A.J., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2004) Hyperbaric oxygen increases network excitability by affecting both intrinsic membrane properties and synaptic transmission. Abstract submitted for Society of Neuroscience, in press.

2. Garcia III, A.J., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2004) Hyperoxia increases neuronal excitability by affecting membrane properties and excitatory input within the CA1 hippocampus. Abstract presented at 2004 Experimental Biology meeting, Washington D.C., FASEB Journal, 18(5): A1058.

3. Potter, S.J., Putnam, R.W., and Dean, J.B. (2004) Decreasing oxygen causes an acidification in neurons from respiratory regions of the medulla. Abstract presented at 2004 Experimental Biology meeting, Washington D.C., FASEB Journal, 18(5): A1058.

4. Dean, J.B. (2004) Hyperoxia and respiratory control: interactions between PO_2, pH and reactive oxygen species. Abstract presented at 2004 Experimental Biology meeting, Washington D.C., FASEB Journal, 18 (5): A1058.

5. Mulkey, D.K., Ritucci, N.A., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Reactive oxygen species (ROS) and chemical oxidants decrease intracellular pH (pH_i) of solitary complex neurons in rat brainstem slices. Abstract presented at 2003 Experimental Biology meeting, Washington D.C., FASEB Journal, 17:A15 (#58.11)

6. Garcia III, A.J., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2003) Oxidative stress induced by hyperbaric oxygen increases the synaptic response of CA1 neurons in the rat hippocampus, *FASEB Journal*, 17:A71 (#78.1)

7. Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2002) Chemical oxidants decrease intracellular pH (pH_i) of solitary complex neurons in rat brainstem slices, *Free Radical Biology and Medicine*, 33:S432 (#386)

8. Garcia III, A.J., Ritucci, N.A., Putnam, R.W., Henderson III, R.A., and Dean, J.B. (2002) The effects of hyperbaric oxygen on intracellular pH of neurons. *Free Radical Biology & Medicine,* 33:S435 (#399)

9. Mulkey, D.K., Henderson III, R.A., Putnam, R.W., and Dean, J.B. (2002) High levels of oxygen selectively stimulate CO_2 chemosensitive neurons in the solitary complex in rat brainstem slices, *FASEB Journal,* 16: A812

10. Garcia III, A.J., Henderson III, R.A., and Dean, J.B. (2002) Acute exposure to hyperbaric oxygen stimulates firing rate of CA1 neurons of the hippocampus, *FASEB Journal*, 16: A1168